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FOOD ACCESSORY FACTORS IN BACTERIAL GROWTH

III. FURTHER OBSERVATIONS ON THE GROWTH OF PFEIFFER'S BACILLUS (B. INFLUENZAE)

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In a previous article,[1] I called attention to the rôle of two substances in the growth of Pfeiffer's bacillus; one, hemoglobin or a derivative and the other, a substance obtainable from plant tissues (carrot, potato), animal tissue, bacteria, yeasts, etc. There are two methods by which these processes have been studied—one, by adding the substances in question to plain blood agar or broth in test tubes and observing the growth after inoculation with Pfeiffer's bacillus; the other by making a poured blood unheated or heated) agar plate, seeding heavily with Pfeiffer's bacilli and then inoculating here and there with bacteria, yeasts, pieces of tissues, etc. About the latter, Pfeiffer's bacilli will develop large or "giant" colonies, in this manner forming a cluster of colonies about the central foreign colony or tissue and known as the "satellite" phenomenon.[2] Further experiments have been made as to the mechanism and the nature of the substances involved in the growth processes of this organism which I wish to report now.

In the following experiments at least two strains of Pfeiffer's bacilli were used; one isolated from a pneumonic lung during the 1919 epidemic, the other from the spinal fluid of a case of so-called influenzal meningitis in a child. No differences of behavior were noticed between these organisms. In certain experiments other respiratory strains of Pfeiffer's bacilli were used with comparable results.

Blood mediums when moderately heated will give a profuse growth of Pfeiffer's bacillus; when heated in the autoclave for 30 minutes it yields practically no growth. The autoclaved medium now may be made to yield excellent growth by adding thereto certain substances which in themselves do not allow growth when added to plain medium.

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[1] Jour. Infect. Dis., 1917, 21, p. 392.
[2] Davis, D. J., Ibid, p. 178.

Such substances include tissues of various kinds, or their extracts or filtrates, such as carrot, potato, and animal tissues. However, when these tissues or their extracts are heated to the boiling point for a time (1 to 2 hours) or are autoclaved they will no longer activate the autoclaved blood. Table 1 illustrates these points.

TABLE 1

GROWTH OF PFEIFFER'S BACILLUS

Plain medium + autoclaved blood	=	0
Plain medium + carrot or potato filtrate	=	0
Plain medium + autoclaved blood + carrot or potato filtrate	=	+++
Plain medium + autoclaved blood + autoclaved filtrate	=	0
Plain medium + washed heart muscle (guinea-pig)	=	0
Plain medium + autoclaved blood + heart muscle	=	+++
Plain medium + autoclaved + autoclaved heart muscle	=	0

Various plant tissues may be used in this experiment and also various animal tissues, such as liver, heart muscle, kidney, brain, spleen, etc. There is an advantage in using plant tissues because they contain no hemoglobin. However, in using animal tissues for activating purposes, by washing small pieces for a long time one can remove this substance. Furthermore by employing the satellite test one can observe the activating effect even on medium containing unheated hemoglobin, so there is no doubt but that animal tissues behave in the same way as plant tissues.

In addition to the plant and animal tissues, various lower organisms may be used in the same manner, either in the form of suspensions or as extracts or filtrates (Berkefeld). Table 2 shows this fact.

TABLE 2

GROWTH OF PFEIFFER'S BACILLUS

Plain medium + autoclaved blood	=	0
Plain medium + bacteria	=	0
Plain medium + autoclaved blood + B. coli	=	+++
Plain medium + autoclaved blood + B. coli filtrate	=	+++
Plain medium + autoclaved blood + heated B. coli (60 C.-30 m.)	=	+++
Plain medium + autoclaved blood + heated B. coli (100 C.-5 min.)	=	+++
Plain medium + autoclaved blood + heated B. coli (autoclaved) (30 min.)	=	0

In this experiment other organisms, like staphylococci, streptococci, sporotricha, blastomycetes, yeasts, etc., may be used, little difference being noted, provided, of course, the reaction is not appreciably altered or is properly adjusted. It will be seen that here again bacteria and other organisms or their filtrates activate the autoclaved blood medium.

By heating the organisms this activating power is gradually reduced so that exposure to the temperature of the autoclave for 30 minutes will cause it to disappear entirely.

It seems clear that we are concerned with two substances, and we may now in the light of these experiments examine further into the behavior of Pfeiffer's bacillus grown on blood and serum medium. Table 3 reveals the facts in condensed form.

TABLE 3

GROWTH OF PFEIFFER'S BACILLUS

Plain medium + crystallized hemoglobin	=	+ (slight)
Plain medium + unheated blood	=	+ (slight)
Plain medium + blood (heated to 60 C. for 5 hours)	=	+++
Plain medium + blood (heated to 100 C. for 5 minutes)	=	+++
Plain medium + blood (autoclaved 30 minutes)	=	0
Plain medium + autoclaved blood + serum	=	+++
Plain medium + autoclaved blood + autoclaved serum	=	0
Plain medium + serum	=	0

From this experiment it is seen that crystallized hemoglobin or ordinary fresh blood plus plain medium is not a good medium though definite growth will occur. When corpuscles are heated to a certain point, however, their value to a medium is markedly enhanced. When heated beyond this point their value is destroyed.

Serum, when added to autoclaved blood medium, will reactivate it promptly, yielding a medium very favorable for growth. Pure serum alone added to plain medium does not yield a growth. Ascitic fluid, if fresh and of high specific gravity, behaves like serum but when of low specific gravity or old it has little or no action. When heated to boiling for 2 hours or autoclaved for 30 minutes the reactivating power of serum and ascites fluid is destroyed. Evidently, then, the serum and the ascites fluid behave quite like plant and animal tissues and also like bacteria and their filtrates referred to.

When ordinary unheated blood (defibrinated or whole blood) is added to plain medium the growth though definite is not abundant. When heated to 55 C. even indefinitely growth also is slight or at times absent. At 60 C. growth is not profuse unless this temperature is applied from 2 to 5 hours. If continued for 2 to 3 days no growth results. At 100 C. a few moments' exposure or simply bringing the medium to this temperature is sufficient to allow profuse growth and exposure for 1 to 2 hours destroys its growth producing value. At 120 C. (autoclave) a few minutes' exposure of the blood mediums

renders it valueless. Thus with increasing temperature the time necessary to obtain a favorable medium becomes less and less and also with increasing temperature the time necessary to destroy its growth value becomes gradually less.

These facts are interpreted as meaning two things. In the first place, the hemoglobin in itself is not a good medium for Pfeiffer's bacillus, perhaps will not support growth at all, and only when it has been changed by heat to hematin or some closely related derivative can it cooperate with a second substance in the blood. This change of hemoglobin appears to take place slowly at 60 C. but more rapidly at higher temperatures. This amount of heat as shown, however, does not destroy the second substance in the blood or the serum; therefore we have the two substances within certain ranges of heating operating together and yielding a profuse growth. If the heating is continued, the second substance is destroyed and no growth takes place without reactivation.

A medium in the form of albumin or peptone appears to be necessary for, as I have shown in previous papers, hemoglobin or hematin alone does not support growth of this organism. The heat resistant substance appears to be hematin or hemin since the action of heat on hemoglobin results in the formation of these substances.

One other point should be mentioned. At or even below about 55 C. blood is coagulated and becomes chocolate in color. However, such blood medium, even though heated for many days (3 weeks), does not yield a favorable medium. When activated with sterile carrot juice the growth is profuse. Blood medium heated at 60 C. or above for 2 or 3 hours yields a good growth without the addition of carrot or tissue juice. My interpretation of this fact is that the temperature of 55 C. or thereabouts is not sufficient in a certain time to cause the change in the hemoglobin resulting in the formation of the derivatives necessary to maximum growth. On the other hand, this temperature continued long enough renders inactive the second substance. There is therefore this interval in the heating of blood in which a profuse growth does not result. Beyond this temperature the hemoglobin is rapidly changed, and heating at the boiling point or at autoclave temperature for hours does not destroy the heat resistant substance (hematin) formed.

As to the second substance: Its heat relation has already been presented. It readily passes through Berkefeld filters and appears to be a product intimately related to living cells. The question as to the

possible relation of this body to growth factors or vitamines naturally arises here. It has been discussed by me in a previous paper [1] and, as then pointed out, there are features about it that suggest that we are dealing with a body of that nature. However, so little is known of the real character of these substances and the criteria for their identification are so indefinite, that little more can be done now to raise the question. I think that both of these bodies may be spoken of as growth or food accessory factors for this organism, using the term in the sense that growth processes depend on them. Whether the mechanism here involved is the same as the mechanism of vitamines in animal growth is not known. However, through the study of such bodies which, as I have detailed, are found in bacteria, yeasts and other tissues, we may be able to throw light on the real mechanism by which vitamines operate. Possibly the ultimate source of these substances may be found in the realm of these lower bacterial organisms. As pointed out,[1] this phenomenon, so far as the growth of Pfeiffer's bacillus is concerned, would seem to center about the metabolism of iron, and this would suggest that the processes are in the main concerned with oxygen or its transfer.

Other agents appear to be able to alter hemoglobin in the same way as heat. The decomposition or digestion of blood by bacteria of various kinds, if not prolonged, yields a product which gives abundant growth. This may be shown by adding putrified blood filtrate to medium in a test tube and inoculating with Pfeiffer's bacilli. If the decomposition of the blood has gone on for a long time (2 to 3 weeks by B. coli for example) no growth of Pfeiffer's bacillus will result on medium to which it has been added. Such medium can be reactivated, however, by adding to it fresh carrot or potato juice or fresh unheated blood, serum or animal tissue. The so-called peptic digest used by Fildes [3] no doubt contains the same substances, the pigment portion being reactivated by the supernatant fluid.

It was shown many years ago by Ghon and Preyss [4] that pure hematin alone would not support the growth of this bacillus but when used on hematin medium with another organism, good growth would result. This has been confirmed by others, including myself, and is in entire accord with the observations detailed now on heated hemoglobin, assuming that hematin results during this process. Hemin also

[3] Brit. Jour. Exper. Path., 1921, 2, p. 16.
[4] Centralbl. f. Bacteriol., 1902, 32, p. 96.

is stated by Olsen [5] to support growth along with other organisms and this substance, too, may result during the process of heating. I have not tested this point myself.

I have attempted to activate many iron and other compounds with substances like carrot juice and bacteria, but I have not been able consistently to do so. The iron derivatives of hemoglobin appear to be the only ones that will so react. I have gone into this point in considerable detail in another paper,[2] and I shall discuss it here no further than to state that for this purpose tests may be readily, and I think delicately made by the plate method used for determination of satellitism.

The relation of this process to the guaiac reaction has been discussed recently by Olsen,[5] who points out that a parallelism exists between the ability of Pfeiffer's bacillus on medium containing hemoglobin and its derivatives and a positive guaiac test. A derivative not containing iron, like hematoporphyrin, will give neither. He does not discuss the question of the reactivation of heated blood by different substances. Fildes [3] also discusses this question but does not explain the fact that many iron and other compounds give a positive guaiac test but do not promote the growth of Pfeiffer's bacillus. He also raises the question as to the possibility of the second substance being a peroxide of such a nature that through the catalytic action of hematin the transfer of oxygen to the bacillus from the peroxide is accelerated. He was led thus to the conclusion to which I was led some years ago [6] through a study of the behavior of blood pigments in high dilutions, namely, that the nature of this process is catalytic.

SUMMARY

Pfeiffer's bacillus grows feebly on mediums containing unheated blood.

Blood mediums heated to 60 C. or higher for definite periods of time yield profuse growth of the bacillus.

Heating in the autoclave (120 C.) for a few minutes or at lower temperatures for longer periods renders blood medium incapable of growing Pfeiffer's bacillus.

This superheated blood medium may be reactivated by adding to it plant, animal and bacterial extracts and filtrates which by them-

[5] Ibid., 1920, 85, p. 12.

[6] Davis, D. J.: Jour. Infect. Dis., 1907, 4, p. 73.

selves do not support growth of this organism. The latter substances lose this property on heating at autoclave temperature for a few minutes or at a lower temperature for longer periods.

The growth process of Pfeiffer's bacillus may be represented thus: plain medium + heat resistant substance (hematin or derivative) + heat labile substance = growth of Pfeiffer's bacillus.